ONE-POT ENZYMATIC TREATMENT OF LIGNOCELLULOSIC BIOMASS FOR BIOENERGY PRODUCTION

A Thesis

by

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Submitted to the Office of Graduate and Professional Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE

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December 2016

Major Subject: Civil Engineering

ABSTRACT

Bioenergy is a form of energy derived from renewable resources, such as lignocellulosic biomass. Lignocellulosic biomass refers to plant material. Many lignocellulosic biomasses considered as waste, such as agriculture waste, forest residues and municipal waste, can be valorized as biofuel. Added benefits from using these lignocellulosic biomasses include minimizing disposal cost and reducing overall greenhouse gas emission. Typical lignocellulosic biomass consists of approximately 40-50% cellulose, 25-30% hemicelluloses, and 15-25% lignin. The biomass is subjected to chemical or physical treatment and enzyme hydrolysis before the deconstructed sugar monomers are utilized for bioenergy production. Because of the tight structure and bonds between the three components of lignocellulosic biomass, physical or chemical treatment is required to swell the plant material to increase accessibility of the enzymes to the cellulose and hemicellulose. Chemical and physical treatments are energy intensive and have the potential to produce inhibitor compounds to reduce efficiency of biofuel production. Cellulose is the main component that is utilized for biofuel production, while hemicellulose and lignin are not depolymerized or utilized in biofuel production. This accounts for approximately 50% waste of biomass.

In this study, we explore the production of high crude lignocellulosic biomass depolymerizing enzymes for efficient deconstruction of lignocellulosic biomass. Specific objective of this study is to investigate complete enzyme deconstruction of lignocellulosic biomass in a single step process. LCD genes were cloned into expression

vectors and transformed into *Escherichia coli* to produce crude enzymes. Crude cellulase, xylanase, and lignin peroxide were produced at 0.18 U/mL, 0.035 U/mL and 0.005 U/mL of activities. Crude cellulase and xylanase were demonstrated to deconstruct lignocellulosic biomass. This demonstrates that lignocellulosic biomass can be destructed to sugar monomers and lignin derivatives by crude enzymes produced by *E. coli*. The depolymerized lignocellulosic biomass can then be utilized by microorganisms to produce bioenergy or triacylglycerol, a precusor to biodiesel.

ACKNOWLEDGEMENTS

I would like to express my upmost gratitude to my committee chair, Dr. Kung-Hui Chu, and my committee members Dr. Bill Batchelor and Dr. Yongheng Huang. I especially want to acknowledge Dr. Kung-Hui Chu for seeing potential in me when I couldn't see it in myself. She gave me the opportunity to mature into the person I am today.

I wish to thank my fellow laboratory mates for taking care of me and our laboratory, where I called home for the last three years. I am proud be given the opportunity to learn along side with them.

Finally, I would like to thank my parents and husband for supporting me and patiently waiting for me to complete my graduate studies. I hope that the knowledge and skills I've gained in my graduate studies will better lives of the people that loves, supports and guides me.

CONTRIBUTORS AND FUNDING SOURCES

This work was supervised by a thesis committee consisting of Dr. Kung-Hui Chu, committee chair, and Dr. Bill Batchelor of the Department of Civil Engineering and Dr. Yongheng Huang of the Department of Biological & Agricultural Engineering. This thesis work was completed independently by the student without additional funding outside of Texas A&M University.

NOMENCLATURE

LCD	Lignocellulosic Depolymerizing
LB	Luria-Bertani
eglS	endo-1,4-β-glucanase (cellulase)
xynA	endo-1,4-β-xylanase (xylanase)
dypB	lignin peroxidase
PCR	Polymerase Chain Reaction
SDS-PAGE	Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis
PBS	Phosphate Buffer Solution
IPTG	Isopropyl β-D-1-thiogalactopyranoside
CMC	Carboxymethyl Cellulose
DNS	3,5-dinitrosalicylic acid
kB	kilobase pair
aa	amino acid
kDA	kilodalton
TAG	Triacylglycerol
U	units
tRNA	transfer RNA

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CHAPTER I

INTRODUCTION

Due to economic and environmental concerns associated with use of fossil fuel, there has been a constant pursuit for a source for renewable energy that is sustainable and economical. Lignocellulosic biomass, the most abundantly renewable raw material on Earth, can serve as an alternative resource to petroleum based energy and/or products[1]. While fossil fuel takes million of years to replenish, lignocellulosic biomass grows in abundance daily. The first generation of biofuel production was characterized by the use of sugars and vegetable oil as feedstock. As a response to the food-for-fuel controversy, the second generation of biofuel production focused on utilizing the inedible portion of lignocellulosic biomass[2]. Agriculture waste, forest residues and municipal waste are examples of lignocellulosic biomass. Producing biofuel from these waste biomass can eliminate cost associated with their disposal and reduce overall greenhouse gas emission.

Lignocellulosic biomass is made of cellulose, hemicelluloses, and lignin. Typical plant material consists of approximately 40-50% cellulose, 25-30% hemicelluloses, and 15-25% lignin[3]. Cellulose and hemicelluloses are sugar polymers that are protected by lignin, which is made complex structure of non-sugar, phenolic alcohol-based polymers. Lignin also protects the plant materials from microbial degradation. Due to the complex and biodegradation resistant nature of the structure, biofuel production from lignocellulosic biomass involves three steps: (1) physical or chemical treatment, (2)

enzyme hydrolysis, and (3) biochemical conversation of released sugars to biofuels. Pretreatment is required to increase depolymerization of lignocellulosic biomass by breaking down of lignin structure, increase surface area and porosity of the material for effective enzymatic hydrolysis. Enzymes hydrolysis entails utilizing commercial enzymes to deconstruct cellulose and hemicelluloses into their constitutive sugar monomers. These released sugars are then available for biofuel-producing bacteria.

Current methods to depolymerize lignocellulosic biomass are costly and inefficient. The cost of enzymes used in enzyme hydrolysis of the biomass accounts for up to 36% of total ethanol production cost or up to $0.78 per gallon of ethanol[4,5]. A viable and economical source of enzymes is required to enable commercial production of biofuels and chemicals from of lignocellulosic biomass. Numerous studies in the last decades focus on exploiting natural or genetically modified microbial production of lignocellulosic depolymerzing (LCD) enzymes. Genetically engineered microorganism, especially *E. coli*, can increase yield and efficiency of lignocellulosic depolymerizing enzymes. These studies are necessary to further advancement the biofuel and chemical production from lignocellulosic biomass. In this study, the potential to depolymerize lignocellulosic biomass through LCD enzymes produced by genetic engineering of *E. coli* was investigated.

The **goal** of this research is to apply genetic engineering to produce crude LCD enzymes to efficiently depolymerize lignocellulosic biomass. To achieve this goal, three tasks were identified and outlined below.

Objective: To reduce biofuel production cost through improved depolymerization of lignocellulosic biomass using high crude lignocellulosic degrading enzymes.

Hypothesis: Improved depolymerization of lignocellulosic biomass can be achieved through a one-pot enzymatic hydrolysis with crude enzymes-lignin peroxidase, cellulase, and xylanase. Crude enzymes can be produced by over expression of proteins in genetic engineered *E coli*.

Task 1: Clone lignocellulosic depolymerizing genes into plasmid and transformation of recombinant plasmid into *E. coli*

Task 2: Induce expression of lignocellulosic depolymerizing genes in *E. coli*

Task 3: Determine sugar released from one-pot enzymatic hydrolysis of lignocellulosic biomass using crude enzymes

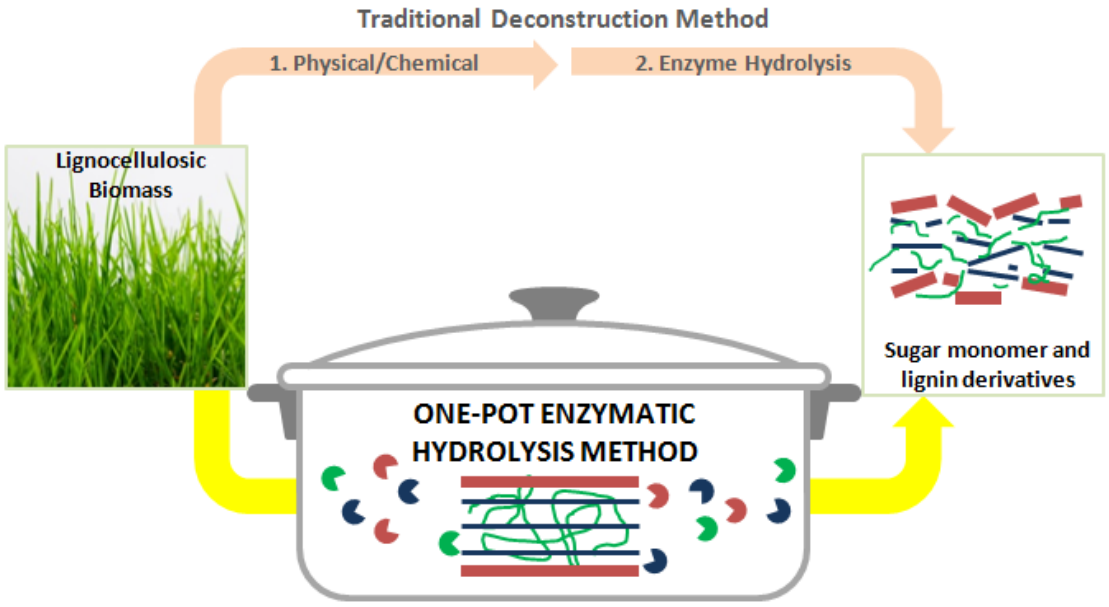


Figure 1. Diagram of proposed one-pot enzymatic hydrolysis of lignocellulosic biomass

This thesis is formatted similarly to that of a manuscript for easy future submission to a peer-reviewed journal. There are 4 chapters and 1 appendix. Chapter 1 gives an overview of scope of this study, including overall goal, hypothesis and tasks of this study. Materials and methods are described in Chapter 2. Results and discussion is described in Chapter 3. Finally, conclusion and suggestion for future studies are in Chapter 4. Literature review on selective topics, including lignocellulosic biomass, pretreatment and enzymatic hydrolysis, hemicelluloses and lignin utilization, and consolidated misprocessing of lignocellulosic biomass, is available in Appendix A.

CHAPTER II

MATERIALS AND METHODS

Strains, plasmid, and growth conditions

Lignocellulosic degrading genes were selected from *Bacillus subtilis* 168 and *Rhodococcus jostii* RHA1. Both strains were maintained on LB medium. The plasmid pET11a was used as the expression vector. The recombinant plasmids were transformed into NEB 5-alpha Competent *E. coli* (High Efficiency) strain (New England Biolabs, Ipswich, MA), *E. coli* BL21 (DE3), and *E. coli* BL21-CodonPlus(DE3)-RIPL (Agilent, Santa Clara, CA). These strains were maintained on Luria-Bertani (LB) medium, supplemented with 100mg/L ampicillin. *E. coli* BL21 CodonPlus (DE3)-RIPL required the addition of 50mg/L chloraphenicol to its growth medium.

Chemicals and lignocellulosic biomass

Genomic DNA was extracted using FastDNA SPIN KIT (MP Biomedicals, Santa Ana, CA). Preparation of the plasmid pET11a and LCD genes was achieved using restriction enzymes (New England Biolabs, Brookfield, WI) and PCR primers (Integrated DNA Technologies, Coralville, Iowa). DNA manipulation was achieved through In-Fusion Cloning Kit (Clontech, Mountain View, CA), Gel Extraction Kit and Miniprep Kit (Qiagen, Hilden, Germany).

LB medium and xylose were purchased from Thermo Fisher Scientific. Isopropyl-D-thiogalactopyranoside (IPTG) was purchased from MP Biomedicals.

Cellulase from *Aspergillus niger* was purchased from TCI America (Portland, OR). Xylan was purchased from Carbonsynth (Berkshire, United Kingdom). All other chemicals were purchased from Sigma-Aldrich (St. Louis, MO). The lignocellulosic biomasses used in this study were obtained locally.

Overview of experimental approach

This research consisted of three tasks to achieve the research goal described above. Task 1 entailed the cloning of selected LCD genes into an expression vector and transformation of the recombinant plasmid into the expression host, *E. coli*. Task 2 determined the amount of functioning crude enzymes produced by recombinant plasmids. Task 3 evaluated the degree of lignocellulosic degradation by the crude enzymes during enzymatic hydrolysis.

Experimental approach

Task 1. Clone lignocellulosic depolymerizing genes into plasmid and transformation of recombinant plasmid into *E. coli*

Task 1a: Select lignocellulosic degrading genes from bacterial strains and expression plasmid and host

Genes encoding cellulase and xylanase producing genes were identified in *B. subtilis* 168 and lignin peroxidase producing genes were recently identified in *R. jostii* RHA1[6]. These LCD genes were cloned into the expression vector, pET11a. The recombinant plasmids were transformed into NEB 5-alpha Competent *E. coli* (High

Efficiency) strain for amplification and maintenance of recombinant plasmids. The recombinant plasmids were transformed into chemical competent *E. coli* strain BL21 (DE3) and *E. coli* BL21-CodonPlus(DE3)-RIPL for expression of LCD genes.

Task 1b: Genomic DNA isolation and PCR amplification

DNA was extracted from *B. subtilis* 168 and *R. jostii* RHA1 grown to stationary phase. Cellulase (eglS) and xylanase (xynA) genes were amplified from B. *subtilis* 168 by polymerase chain reaction (PCR) using the genomic DNA as template. Lignin peroxidase (dypB) was amplified in the same manner from *R. jostii* RHA1. Forward and reverse primers for these LCD genes were designed using Primer Design tool for In-Fusion® HD Cloning Kit (http://www.clontech.com). Each LCD gene was designed to be inserted into individual pET11a vectors between the Nhel-Ndel cloning sites.

Table 1. Selected lignocellulosic degrading genes

Gene	Function	Origin	Size (kB)	Size (aa)	MW (kDa)
eglS	endo-1,4-β-glucanase (**cellulase**)	*Bacillus subtilis* 168	1.5	500	55
xynA	endo-1,4-β-xylanase (**xylanase**)	*Bacillus subtilis* 168	0.65	200	23
dypB	**Lignin Peroxidase**	*Rhodococcus jostii* RHA1	1	350	38

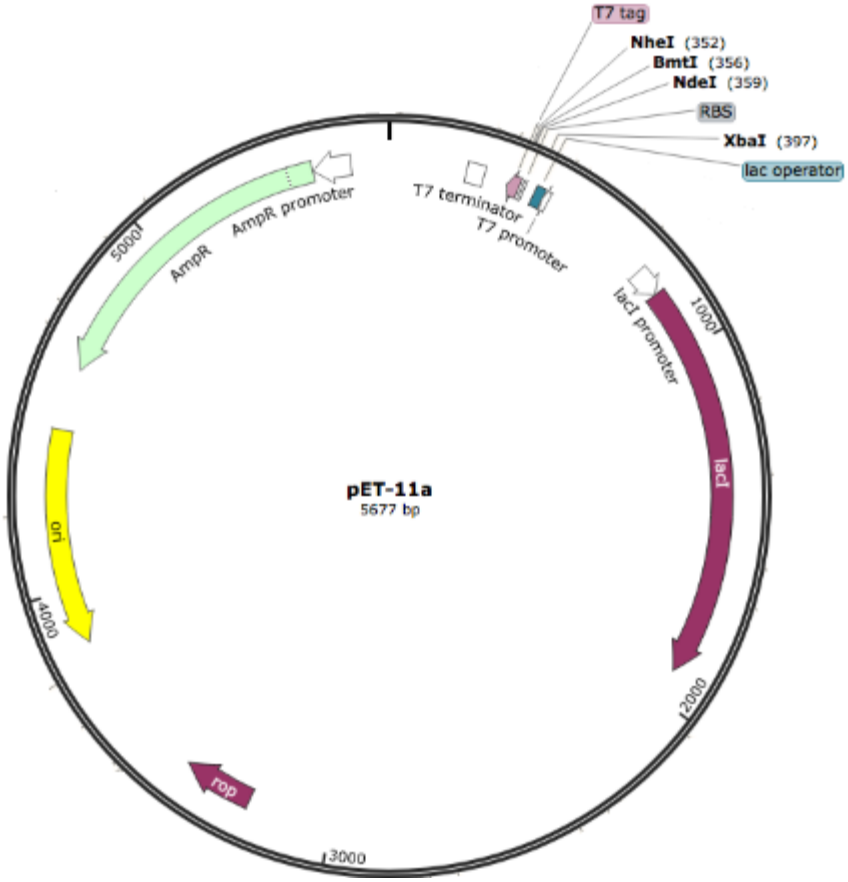


Figure 2. Map of pET11a plasmid[7]

The 25 uL PCR reaction contained 12.5 µL of 2X CloneAmp Hifi PCR Premix, 1 µL of 10 µM forward and reverse primer, 3 µL of 70-80 ng/µL template DNA. PCR reaction was carried out by denaturing at 50°C for 2 minutes, followed by 35 cycles at 98°C for 10 seconds, 55°C for 15 seconds, and 72°C for 1.5 minutes (eglS and xynA) or 1 minutes (dypB), with final incubation at 72°C for 10 minutes for final extension. The 5 µL of PCR products were observed by electrophoresis on 0.9% agarose gel. Cellulase and xylanase DNA bands were purified using Clone Enhancer. Lignin peroxidase DNA

band was purified using Gel Extraction Kit. DNA concentration was quantified using NanoDrop Lite spectrophotometer (Thermo Fisher Scientific, Waltham, MA).

Task 1c: Cloning of lignocellulosic degrading genes into pET11a and transformation of recombinant plasmids into E. coli BL21 (DE3)

Restriction digest of plasmid pET11a was prepared by adding each 1 μL (20 units) of Nhel and Ndel restriction enzyme, 10 μL of 90 ng/μL template DNA, 5 μL 10X CutSmart Buffer. The 50 μL digestion mixture was incubated at 37°C for 3 hours. The linearized plasmid was observed by agarose gel electrophoresis and purified by Gel Extraction Kit. The purified LCD genes were ligated into Nhel-Ndel restriction sites using In-Fusion Cloning Kit. The 10 μL ligation mixture for cellulase and xylanase contained 2 μL of 5X In-Fusion Cloning HD Enzyme Premix, 3 uL of 90 ng/uL linearized pET11a, and 1 uL of purified PCR fragment. Due to low yield of purified PCR product of dypB, the ligation mixture for dypB was doubled containing 4 uL 5X In-Fusion Cloning HD Enzyme Premix, 3 uL of 90 ng/uL linearized pET11a, and 8 uL of 8 ng/uL of purified PCR fragment. The ligation mixtures were incubated at 50°C for 15 minutes.

The recombinant plasmid DNA were transformed into NEB 5-alpha Competent *E. coli* (High Efficiency) strain and screened on LB agar contained 100mg/L ampicillin. Verification of recombinant plasmid was achieved by extracting the plasmid DNA and digesting by unique restriction enzyme at 37°C for 1 hour of cultures grown overnight. The plasmid DNA were cut into 1-3 uniquely sized fragment by BamH1 and EcoRV

restriction enzymes and visualized by agarose gel electrophoresis. The plasmids with correctly size fragments were sequenced (Eton Bioscience, San Diego, CA) over the entire LCD gene and analyzed by APE, a plasmid editor tool (http://biologylabs.utah.edu/jorgensen/wayned/ape). The correct recombinant plasmids were then transformed into chemically competent cells of *E. coli* BL21 (DE3)[8] and *E. coli* BL21-CodonPlus(DE3)-RIPL.

Task 2. Induce expression of lignocellulosic depolymerizing genes in *E. coli*

Task 2a: Expression of LCD genes and visualization of protein on SDS-PAGE

To express the three recombinant lignocellulosic degrading genes, the freshly transformed *E. coli* BL21(DE3) and *E. coli* BL21-CodonPlus(DE3)-RIPL cells were grown by inoculating 100 mL growth medium with overnight culture to an initial OD_{600} of 0.05. The cultures were incubated at 37°C at 200 rpm. 1.0 mM IPTG was added to the cultures when the OD_{600} reached 0.4 to 0.7 to induce the expression of the LCD genes. The cultures were harvest after another 2 hours of incubation. Expression of lignin peroxidase and xylanase genes were also determined in *E. coli* BL21-CodonPlus(DE3)-RIPL when induced at 0.1, 0.4 and 1.0 mM IPTG and incubated for an additional 2 and 4 hours. Cells were harvested by centrifugation at 10,000 rpm for 10 minutes at 4°C. The cell pellets were resuspended in 2 mL Phosphate Buffer Solution (PBS) (pH 7.0). LCD proteins of cell cultures (ODunit of 0.15 to 0.25) were visualized by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). SDS-PAGE was performed on 14% Tris-Glycine Gel in the Surelock Minicell apparatus (Thermo Scientific, Waltham,

MA). The electrophoresis was conducted at 90 voltage for 2.5 hours. The gel was then stained with Coomassie Blue dye for 4 hours and destained for an hour.

Task 2b: Determine enzyme activity using colorimetric assays

Protein was measured from cell cultures lysed by sonication and freeze-thaw methods. After appropriate incubation after induction of recombinant protein, cell cultures were pelleted and resuspended in 1/50 volume of in PBS (pH 7.0) and stored at -20°C. To lyse the cells, the cultures were again subjected to additional freezing at -80°C for two minutes and thawing at 37°C for 15 minutes for three cycles before resuspending in PBS at pH 7.0. Sonication were carried out in 1 mL of cell cultures at OD_{600} of 1.0 in 3 rounds of 20 seconds sonication (50% amplification) and 20 seconds ice bath intervals. Cell fraction and supernatants were tested for enzyme activities.

Cellulase activity was determined by 3,5-Dinitrosalicylic acid (DNS) Reducing Sugar Method[9], using D-glucose as the standards. The substrate 1% Carboxymethyl cellulose (CMC) prepared in 0.05 M Na-citrate buffer (pH 6.0). Positive enzyme control was measured by adding 1 and 10 μg/mL pure cellulase from *Aspergillus niger* prepared in 0.05 M Na-citrate buffer (pH 6.0). One mL of cell cultures of OD_{600} at 1.0 in PBS (pH 7.0) or 1 mL of pure enzyme was added to 1 mL of 1% CMC in 16mm test tube and incubated at 50°C for 20 minutes. Two mL of modified DNS Reagent (1% 3,5-dinitrosalicylic acid 1% NaOH, 0.2% phenol, and 0.05% sodium sulfite) was added to the test tube. The mixture was placed in a boiling water bath for 15 minutes. One mL of 40% Rochelle salt solution was added to the test tube before cooling down to room

temperature. The mixture was measured colormetrically at OD_{560} using a microplate reader (Tecan GENIOS, Männedorf, Switzerland). One unit (U) of cellulase activity will liberate μmole of glucose per minute at given temperature and pH.

Xylanase activity was also determined by DNS Reducing Sugar Method[10] similar to cellulase, except xylose served as the standards. The substrate 0.05% xylan prepared in 0.05 M Na-citrate buffer (pH 6.0). Positive enzyme control was measured by adding 1 and 10 μg/mL pure endo-1,4-β-Xylanase from *Trichoderma longibrachiatum* prepared in 0.05M Na-citrate buffer (pH 6.0). 200μL of cell culture of OD_{600} at 1.0 in PBS (pH 7.0) or 200μL of pure enzyme was added to 1.8 mL 0.05% xylan in 16mm test tube. The mixture was subjected to the same chemicals reaction and incubation conditions as the cellulase assay. One unit of xylanase activity will liberate μmole of xylose per minute at given temperature and pH.

Lignin peroxidase activity was determined by Azure B Methods[11], using azure B as substrate. The cell filtrates were prepared by sonicating one mL of cell culture of OD_{600} at 1.0 in PBS. The cells were centrifuged at 13000 g for 10 minutes and the supernatant were filtered through 0.2 μm pore size. The reaction was carried out in 1 mL of 125 mM sodium tartrate buffer (pH 3.0), 500 μL of 0.160 mM azure B, 500 μL 2 mM hydrogen peroxide, 500 μL cell filtrate. The reaction was initiated by the addition of hydrogen peroxide. After 10 minutes of incubation at room temperature, the mixture was measured colormetrically at OD_{612} using the microplate reader. One U of lignin peroxidase activity will liberate μmole of 3.4-dimethoxybenzyl alcohol per minute at given temperature and pH.

Task 3. Determine sugar released from one-pot enzymatic hydrolysis of lignocellulosic biomass using crude enzymes

Task 3a: Depolymerization of lignocellulosic biomass

The lignocellulosic biomasses were dried at 50°C for 24 hours and grinded up in coffee grinder[12]. One-pot enzymatic hydrolysis was conducted utilizing crude enzymes produced by *E. coli* BL21 (DE3) and *E. coli* BL21-CodonPlus(DE3)-RIPL with recombinant plasmids. Cells were grown and harvest as stated in Task 2a. Cell pellets were then frozen at -80°C for 2 minutes and thawed at 37°C while shaking at 200 rpm for 10 minutes. After three freeze-thaw cycles, the cell pellets were resuspended in 0.05 M Na-citrate buffer (pH 3.0 for lignin peroxidase and pH 6.0 for cellulase and xylanase) and incubated at 37°C while shaking at 200 rpm for 10 minutes. The cell cultures were then centrifugated at 10000 rpm for 10 minutes (4°C). The supernatant was applied to the biomass as crude enzymes. Crude lignin peroxidase was added to 1 gram of dry, grinded lignocellulosic biomasses. 4 mM H_2O_2 and 1 mM $MnCl_2$ were also added to facilitate and increase activity of the lignin peroxidase[6]. The biomasses were incubated at 30°C at 200 rpm for 24 hours. After 24 hours, the pH of the biomasses was adjusted to 6.0 using 1% NaOH. Crude cellulase and xylanase enzymes were added and the biomasses were incubated at 50°C at 200 rpm for an additional 24 hours. The hydrolysate was separated from the solid biomass via centrifugation at 10000 rpm for 10 minutes (4°C) and tested for reduced sugar concentration.

Task 3b: Determination amounts of reduced sugars from depolymerized biomass

The resulting hydrolysates were tested for reduced sugar concentration by DNS Reducing Sugar Method[9]. Two mL of diluted hydrolysis were added 2 mL of modified DNS Reagent in 16mm test tube. The tubes were placed in a boiling water bath for 15 minutes. One mL of 40% Rochelle salt solution was added to the test tube before cooling down to room temperature and measuring colormetrically at OD_{560} using the microplate reader. Reduced sugar was measured as glucose equivalence.

CHAPTER III

RESULTS AND DISCUSSION

Cloning of LCD genes in pET11a and transformation

Genomic DNA of *B. subtilis* 168 and *R. jostii* RHA1 were measured at 220 ng/uL and 80 ng/uL. The gene of interest amplified by PCR were visualized on gel and purified. Clear bands of 1.5 kilobase pairs (kB) for cellulase and 0.65 kB for xylanase were observed on the agarose gel. Because smear bands were observed for dypB, the 1.0 kB band of PCR product was purified from the agarose gel. The concentration of purified PCR product of dypB was measured at 8 ng/μL, which was low but sufficient for cloning. The concentration of purified pET11a expression vector, linearized at Nhel and Ndel cloning sites, was measured at 15 ng/μL. The LCD genes were cloned into individual pET11a vectors to produce three recombinant plasmids (Figure 3). The series of pET vectors were engineered to promote high-level transcription and translation of inserted genes by the T7 promoter, which was derived from T7 bacteriophage gene 10. The T7 promoter is inactive until induction, during which the inserted gene of interest will be transcribed at a high rate. The recombinant LCD genes can be produced within hours of induction using these pET vectors. Ampicillin resistant gene in pet vectors serves as the selection marker.

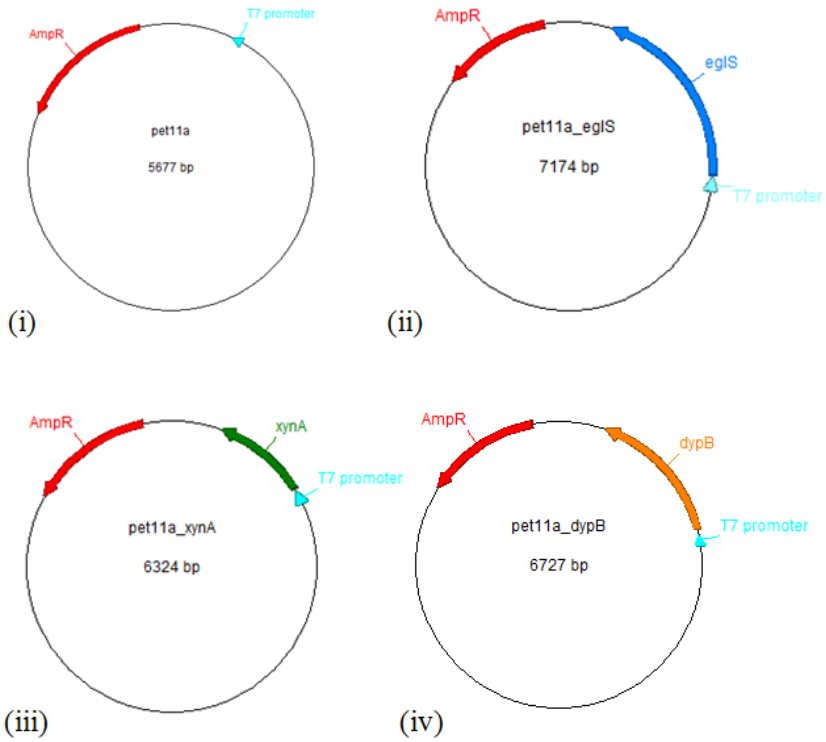


Figure 3. Map of constructed recombinant plasmids pET11a (i), pET11a/eglS (ii), pET11a/xynA (iii), and pET11a/dypB (iv)

After the three recombinant plasmids were transformed into NEB 5-alpha Competent *E. coli*, they were confirmed using two simple methods-agarose gel electrophoresis and DNA sequencing. The plasmid DNA was extracted, cut to unique fragment size with restriction enzymes (Table 2) and visualize on agarose gel (Figure 4). Plasmid DNA from at least one transformant colony was confirmed by sequencing. Each recombinant plasmids with LCD genes were transformed into individual chemically competent cells of *E. coli* BL21 (DE3). When it was discovered that xylanase and lignin peroxidase were produced at low levels in *E. coli* BL21 (DE3), the two recombinant plasmids were transformed into *E. coli* BL21-CodonPlus(DE3)-RIPL.

Table 2. Expected plasmid DNA fragment size from restriction digestion from BamH1 (1 cut) and EcoRV (2 or 3 cuts)

	no cut [kB]	1 cut [kB]	2 or 3 cuts [kB]
pET11a-eglS	3.5	7.0	3.0, 4.0
pET11a-xynA	3.2	6.3	0.8, 1.5, 4.0
pET11a-dypB	3.4	6.7	2.5, 4.2

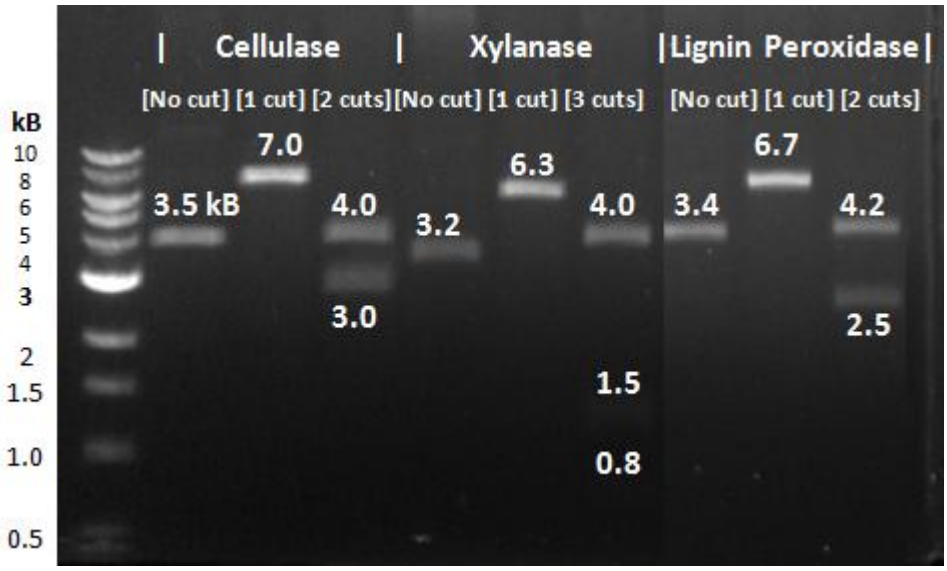


Figure 4. Agarose gel of cut and uncut recombinant plasmids extracted from NEB 5-alpha Competent *E. coli*

Expression of recombinant proteins

To visualize the expression of LCD genes in *E. coli* BL21 (DE3), 1 mM of IPTG were added to mid-log growth cells and incubated for an additional 2 hours at 37°C. The protein profiles of concentrated whole cells cultures were analyzed on SDS-PAGE, as shown on Figure 5. A large 55 kilodalton (kDa) band were observed on the polyacrylamide gel. This indicated significant cellulase enzyme production from *E. coli*

BL21 (DE3), with cellulase as half the total protein. A fainter 39 kDa band for lignin peroxidase was observed, while no distinct 22 kDa band was observed for xylanase.

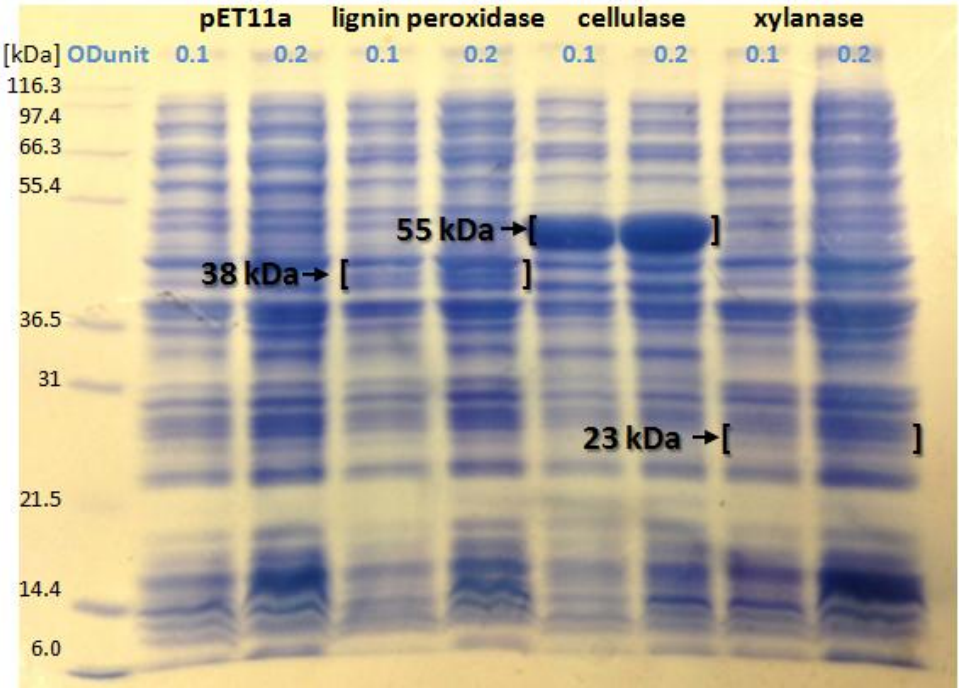


Figure 5. SDS-PAGE analysis of recombinant protein in *E. coli* BL21 (DE3) after 2 hours of induction with 1mM IPTG

Improving expression of recombinant lignin peroxidase and xylanase

After careful literature review, many options were explored to improve the expression in lignin peroxidase and xylanase. Optimizing induction conditions and issues of rare codons in *E. coli* were further studied.

Optimizing induction conditions

The pet expression vector is characterized by the T7 promoter and induction with IPTG. Optimizing induction conditions can yield in higher and more stable protein production. Typically induction is carried out in 0.05 mM-2 mM of IPTG. Lower concentration will facilitate production of target protein in its desired soluble form, while higher concentrations will produce insoluble proteins, or inclusion bodies[13]. Sufficient production of protein is typically observed within a few hour of induction but can be carried out overnight. Longer cultivation at lower temperature, 30°C, can be explored as it also promotes soluble protein expression. Production of recombinant xynA from *Bacillus licheniformis* 9945A cloned in pET22b+ expression vector was previously shown to be optimized at growth conditions of starting pH 7.0 and 37°C and induction conditions of 0.4 mM IPTG for 4 hours[14]. Protein production of lignin perodixase and xylanase were tested at various IPTG concentrations for four hours at 37°C. However, at these conditions, protein production not improved (Figure 6).

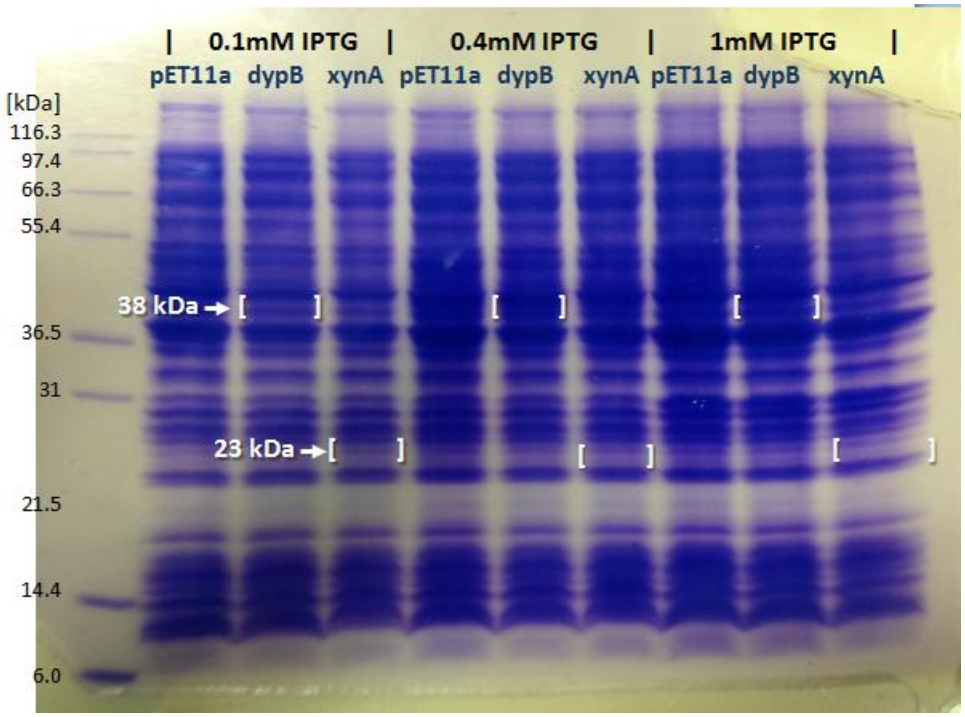


Figure 6. SDS-PAGE analysis of recombinant protein in *E. coli* BL21 (DE3) after 4 hours of induction with 0.1, 0.4, and 1mM IPTG

Low translation due to presence of rare codon

Most amino acids are encoded by more than one codon, but each organism has biased distribution of 61 amino acid codons (Figure 7). When recombinant genes carry animo acids that are rarely encoded in *E. coli*, the animo acid will be encoded by a different codon and results in error in translation. This translation mismatch can be significant over expression of target genes, such as in pET vectors. Analysis of *E. coli* codon usage reveals to lack populations of certain codons. Rare codons in *E. coli* are Arg codons (AGA, AGG, CGG, and CGA), Ile codon (AUA), Leu codon (CUA), Gly codon (GGA) and Pro codon (CCC)[15]. Large presence of rare codons and rare codons found in

clusters or the start of the genes are all strong indications of protein production issues in genes.

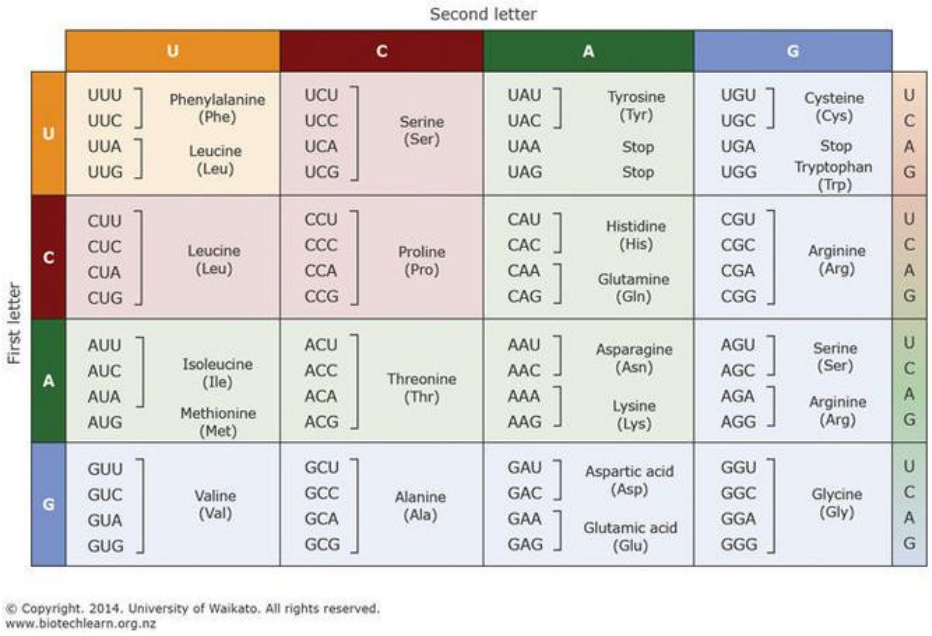
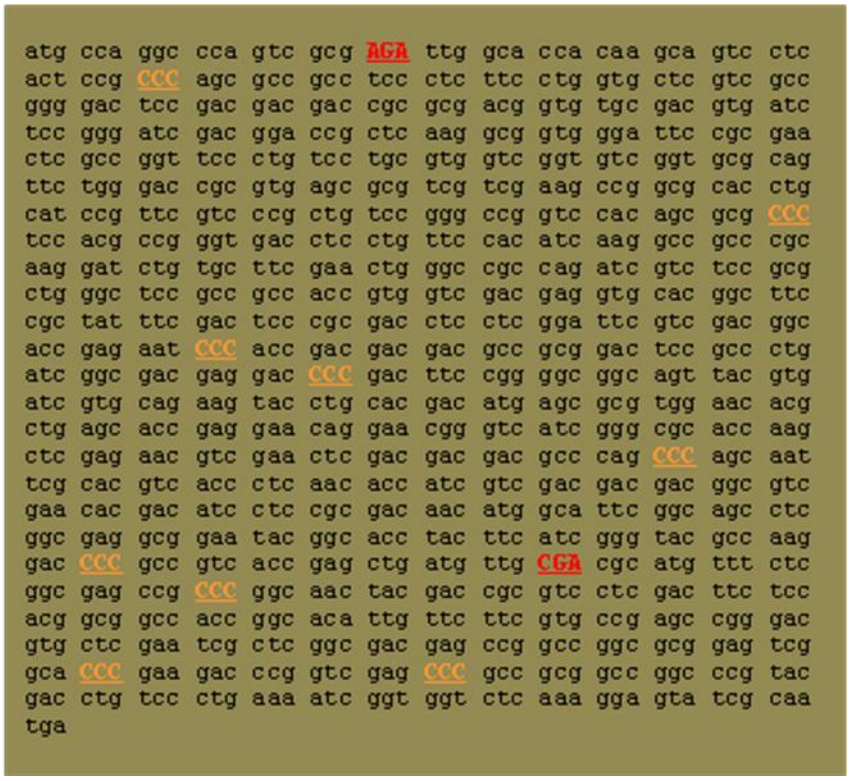


Figure 7. RNA codon table[17]

The three recombinant genes were analyzed for presence of rare codon (http://nihserver.mbi.ucla.edu/RACC), as shown on Figure 8. Lignin peroxidase contains 3% Pro codon CCC alone and Arg codon AGA at the beginning of the gene. Arg codons are the most infrequent of the rare codons. Xylanase has several rare codon clusters at 7% total codons. Studies have shown that xylanase from other microbial strains having issues with expression in *E. coli*, but have been successfully demonstrated[14],[16]. Many of those xylanase genes contained 3-4% rare codons. Cellulase has 2% rare codon, which is much lower percentage of rare codons than the other two LCD genes.

(i)



(ii)

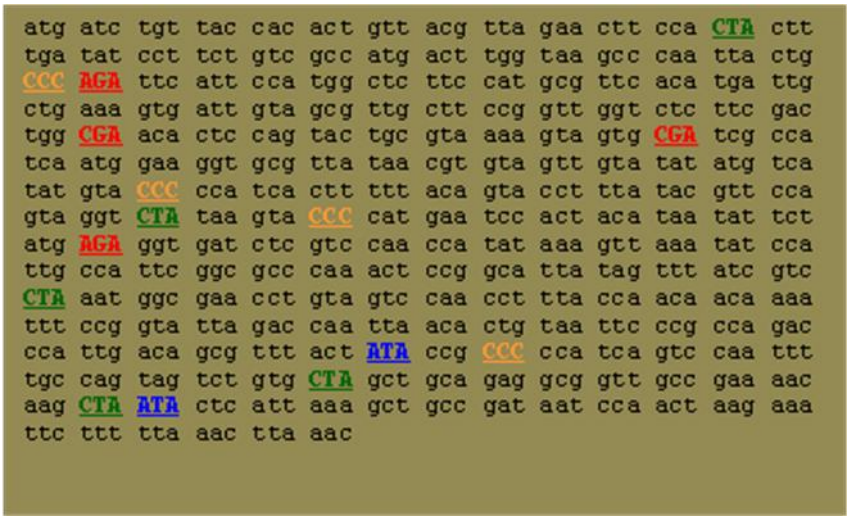


Figure 8. Rare codon analysis of dypB (i), xynA (ii), and eglS (iii)

(iii)

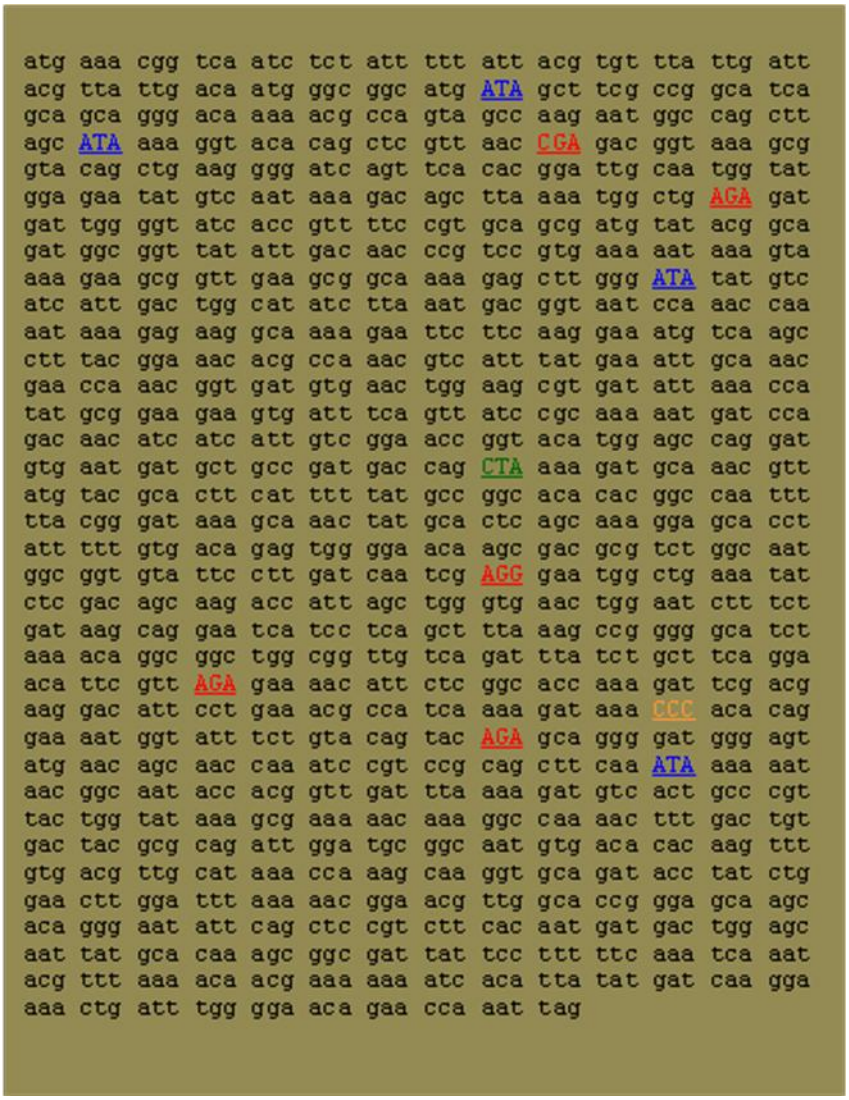


Figure 8. Continued

To minimize the effect of rare codons, the recombinant plasmids were transformed into *E. coli* BL21-CodonPlus(DE3)-RIPL. This engineered strain contains extra copies of genes that encode the tRNAs that most frequently limit translation of proteins in *E. coli*. Availability of these tRNAs allows for higher expression of many recombinant genes that are otherwise poorly expressed in conventional *E. coli* BL21

(DE3) strains. *E. coli* BL21-CodonPlus(DE3)-RIPL contains more Arg (AGA, AGG), Ile (AUA), Pro (CCC), Leu (CUA). Induction conditions tested were 0.1 to 1 mM IPTG for 2 (Figure 9) and 4 hours (Figure 10). SDS-PAGE showed a 39 kB band for lignin peroxidase at higher IPTG concentration, but no xylanase band was observed at 23 kB.

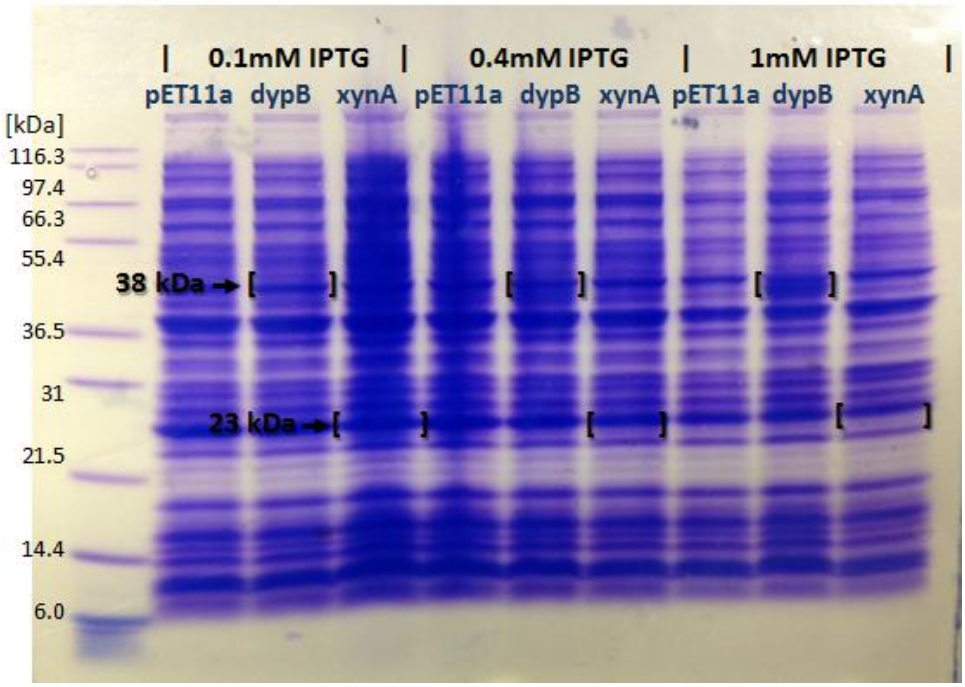


Figure 9. SDS-PAGE analysis of lignin peroxidase and xylanase in *E. coli* BL21-CodonPlus(DE3)-RIPL induced for 2 hours

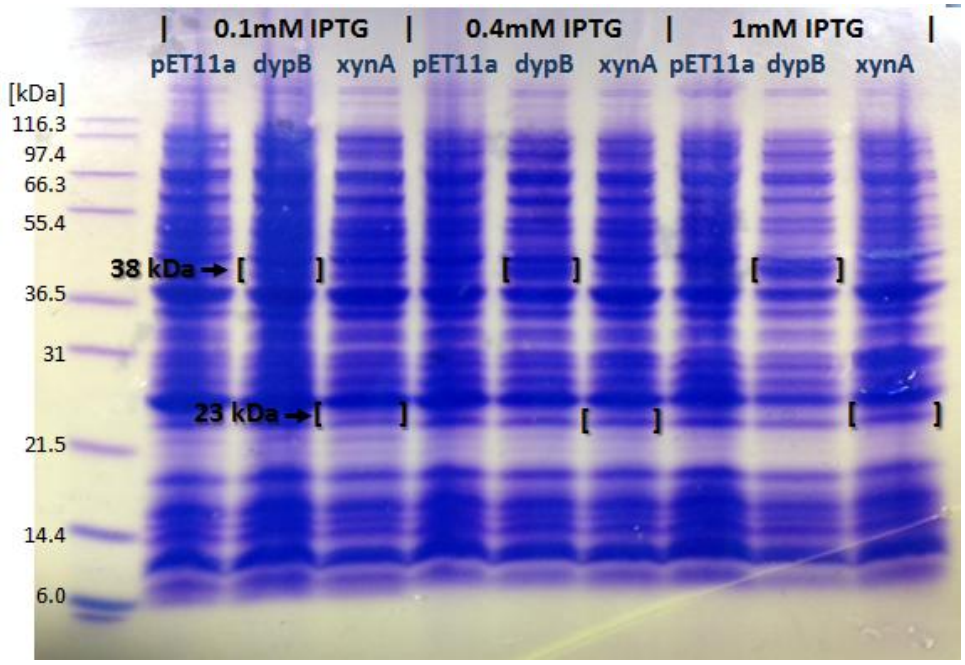


Figure 10. SDS-PAGE analysis of lignin peroxidase and xylanase in *E. coli* BL21-CodonPlus(DE3)-RIPL induced for 4 hours

Although lignin peroxidase production is improved by taking advantage of *E. coli* BL21-CodonPlus(DE3)-RIPL, the amount of protein produced is not comparable to cellulase production in *E. coli* BL21 (DE3). Xylanase activity was measured to determine whether xylanase enzymes were produced or not. Enzyme activity of xylanase indicated low activity (approximately 0.035 U/mL) in *E. coli* BL21-CodonPlus(DE3)-RIPL but no activity in *E. coli* BL21 (DE3) (Figure 11). Xylanase production is too low to visual on the polyacrylamide gels. Low production of xylanase can be overcome by growing larger volumes of cell cultures. The LCD genes expression in *E. coli* BL21 (DE3) and *E. coli* BL21-CodonPlus(DE3)-RIPL will analyzed for protein solubility and

enzyme activity. The cells will be induced with by 1mM IPTG for 2 hours and lysed by freeze-thaw method.

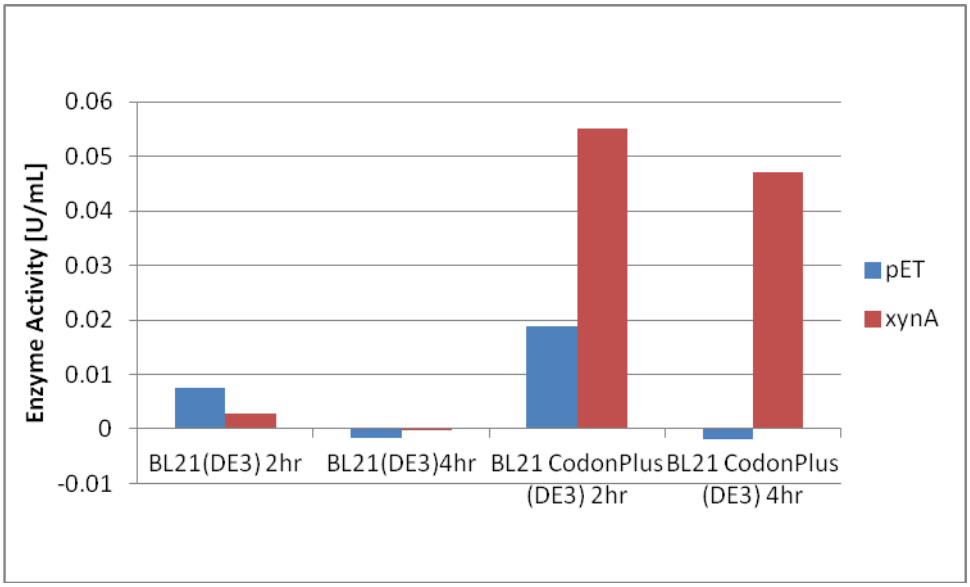


Figure 11. Xylanase activities of whole cells from *E. coli* BL21 (DE3) and *E. coli* BL21-CodonPlus(DE3)-RIPL induced by 1mM IPTG for 2 and 4 hours

Solubility of recombinant proteins

A major concern of high expression of recombinant protein is the aggregation of protein in its insoluble form known as inclusion bodies. The inclusion bodies reduce the functionality of these proteins. This is a concern for cellulase produced from *E. coli* BL21 (DE3). As shown in the SDS-PAGE (Figure 5), cellulase is the most predominant protein in the *E. coli* BL21 (DE3). Solubility of total protein in both *E. coli* strains were measured in cell fraction and supernatant.70-90% of proteins were released in supernatant of cells lysed by sonication. This indicated that inclusion bodies were not a

concern for cellulase proteins produced in *E. coli* BL21 (DE3). Because harvesting large volumes of crude enzymes by sonication is energy intensive and not practical, cells lysis was achieved by freeze-thaw method from here on out. 50% of proteins was released into supernatant when lysed by freeze-thaw method (Figure 12-13) These findings are consistent with a previous study of releasing recombinant protein in *E. coli*[18]. Total protein concentrations were measured at 0.8-1.2 mg/mL for cell cultures of OD_{600} at 1.0 for these *E. coli* strains.

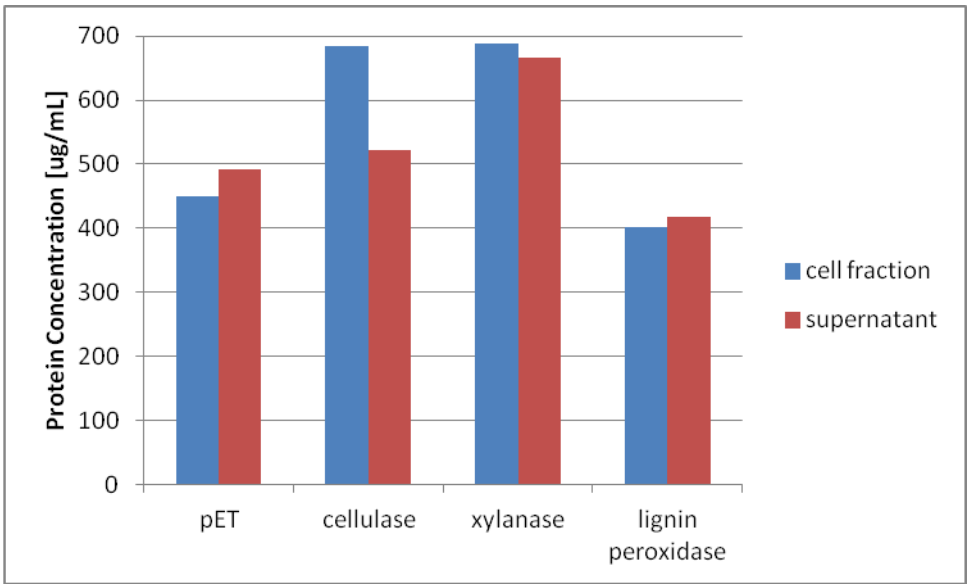


Figure 12. Protein concentration of cell fraction and supernatant of *E. coli* BL21 (DE3)

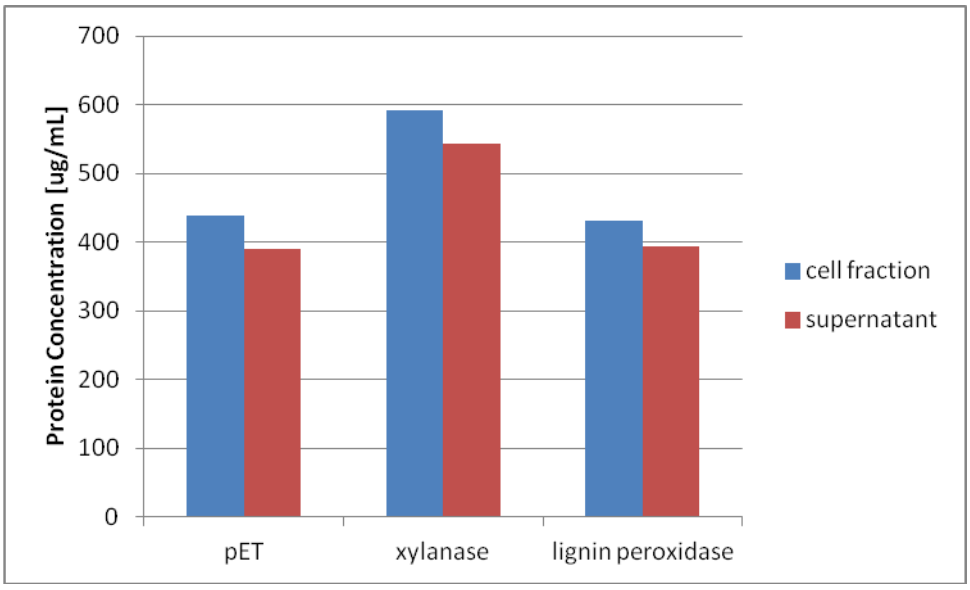


Figure 13. Protein concentration of cell fraction and supernatant of *E. coli* BL21-CodonPlus(DE3)-RIPL

Enzyme activity of recombinant proteins

Solubility is an indication of functional protein but catalytic activity of these enzymes needs to be quantified. Each LCD enzymes were measured for activities from whole cells (OD_{600} at 1.0), cell fraction, and/or supernatant. Cellulase activity was estimated at 0.18 U/mL for whole cells, cell fraction, and supernatant (Figure 14), indicating sufficient cellulase release by freeze-thaw method. Because enzyme activity is a rate, measure as one unit released one μmol of sugar per minute, the enzyme activity of the whole cell is not equal to the summation of the cell fraction and supernatant. Approximately 1 mL of OD_{600}=1.0 of cellulase producing *E. coli* BL21 (DE3) shows similar activity to 0.75 mg of commercial cellulase. One gram of commercial cellulase was estimated at 0.24U/mL.

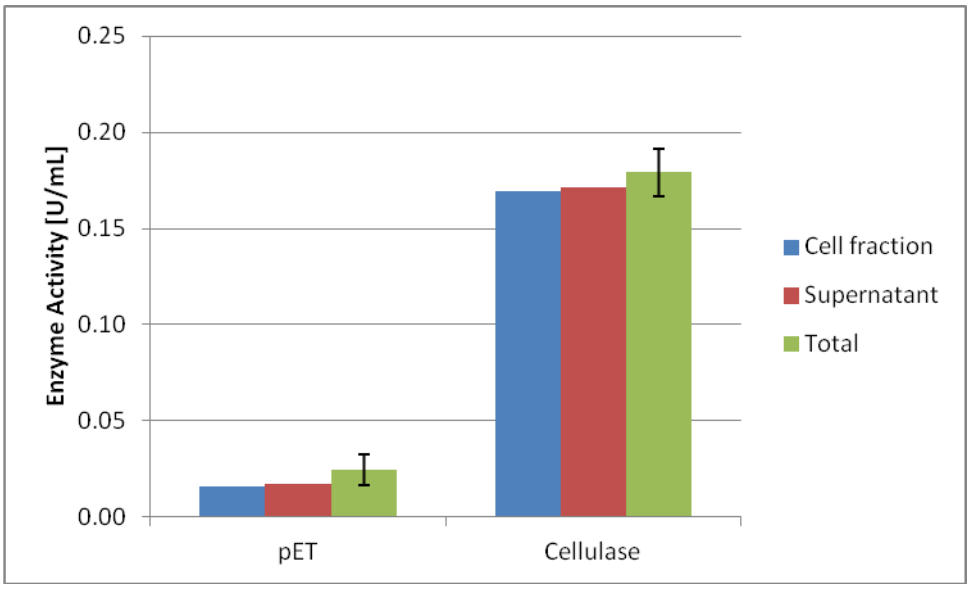


Figure 14. Cellulase activities of whole cells, cell fraction and supernatant from *E. coli* BL21 (DE3)

Xylanase activities of *E. coli* BL21 (DE3) and *E. coli* BL21-CodonPlus(DE3)-RIPL were determined previously in Figure 11. Xylanase activity in *E. coli* BL21-CodonPlus(DE3)-RIPL estimated at 0.035 U/mL, while the original BL21 (DE3) strain did not show any activity. Figure 15 shows that approximately 25% enzymatic activity was observed in supernatant compared to cell fraction when *E. coli* BL21-CodonPlus(DE3)-RIPL cells were lysed by freeze-thaw. This may be an indication of inclusion bodies, but overall enzyme activity is too low to confirm. The low enzyme activities for xylanase are corresponded to the absence of 23 kB protein band on the polyacrylamide gels.

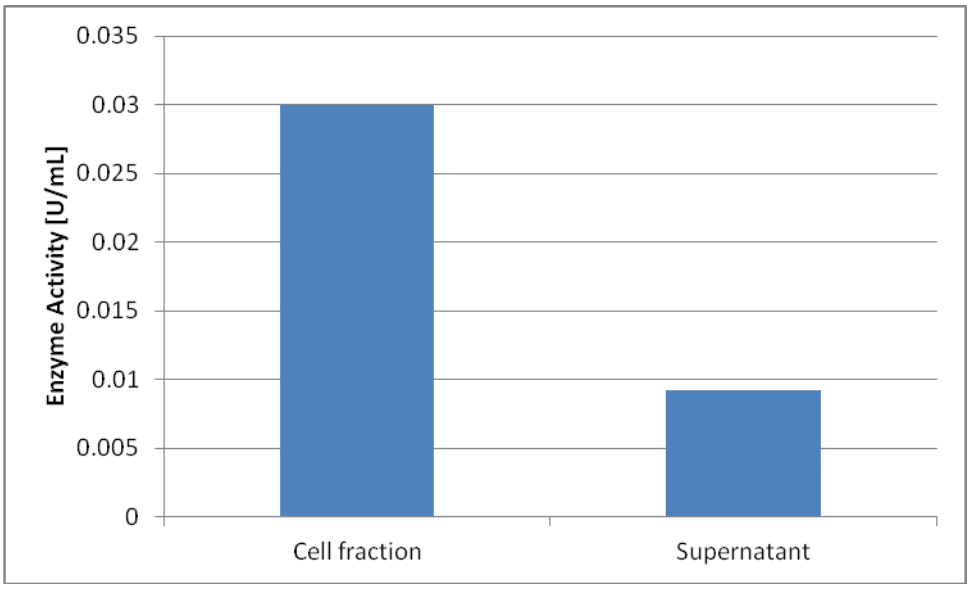


Figure 15. Xylanase activities of cell fraction and supernatant from *E. coli* BL21-CodonPlus(DE3)-RIPL

Lignin peroxidase activity *E. coli* BL21-CodonPlus(DE3)-RIPL supernatant lysed by freeze-thaw methods was estimated at 0.005 U/mL (Figure 16). The significant decrease in optical density in pET control is due to adsorption of substrate to particulates. Low lignin peroxidase activity is consistent with previous studies. Bacterial or fungal lignin peroxidase has very low level of production and activity. Production of lignin degrading enzymes in fungi is typically low and is only trigger by nutrient limited conditions[19]. There is currently no economically viable method to commercialize the production of lignin degrading enzymes, whether it is lignin peroxidase, manganese peroxidase, or laccase.

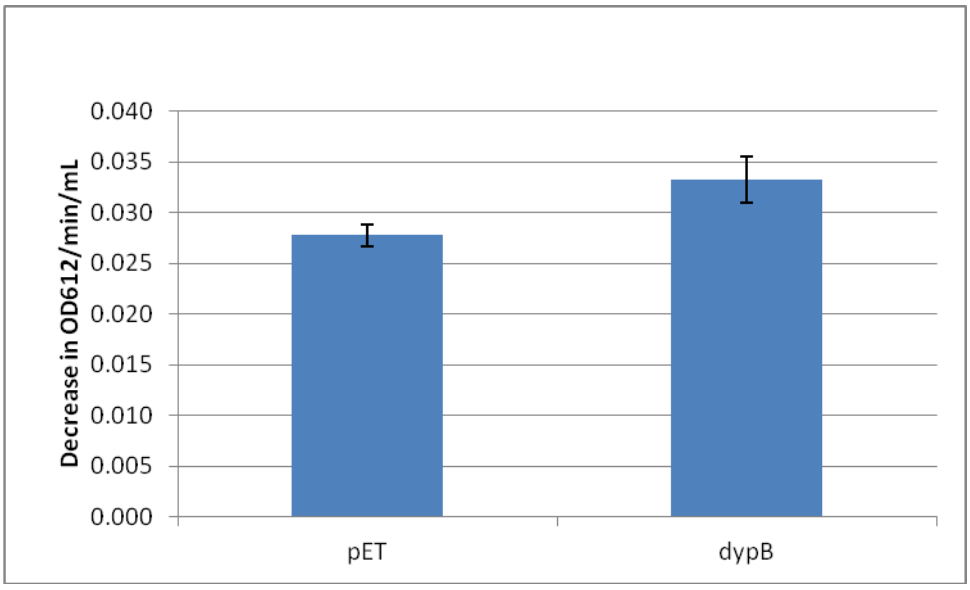


Figure 16. Lignin peroxidase activities of supernatant from *E. coli* BL21-CodonPlus(DE3)-RIPL

Hydrolysis of lignocellulosic biomass with crude enzymes

After verifying production and activities of the three LCD enzymes, the crude enzymes can be applied to various lignocellulosic biomasses for enzyme hydrolysis. Crude cellulase produced from recombinant *E. coli* and purified cellulase was added (8 U/g biomass). Effect of crude xylanase on cellulase was determined, regardless of its low activity. Approximately 1.5 U/g of xylanase was applied to the biomass. Crude lignin peroxidase was not applied in this one-pot method because lignin peroxidase requires oxidation by hydrogen peroxide. When crude lignin peroxidase was added with the one-pot method, there was minimal released sugar measured. The hydrogen peroxide was observed to oxidize any reduced sugar produced by cellulase and xylanase[20]. After initial depolymerization of biomass by lignin peroxidase, the biomass should be washed

before adding cellulase and xylanase. Washing the biomass in between deconstructions causes losses in carbohydrates, an issue with current traditional deconstruction method.

In Figure 17, the crude enzyme cocktail of cellulase and xylanase was only effective on corn husk, at 0.01 g/g biomass. The crude enzymes yielded no additional depolymerization in grass and paper biomass when compared to the blank control. Both grass and paper have much higher hemicellulose and lignin content than corn husk. These lignocellulosic biomasses contain up to 40-50% hemicellulose and 30% lignin[21]. With hemicellulose and lignin not efficiently deconstructing or depolymerizing, cellulase has little access to cellulose for depolymerization. Because of the integrated structure of lignocellulosic biomass, a cocktail of enzymes will demonstrate increase efficiency in deconstruction.

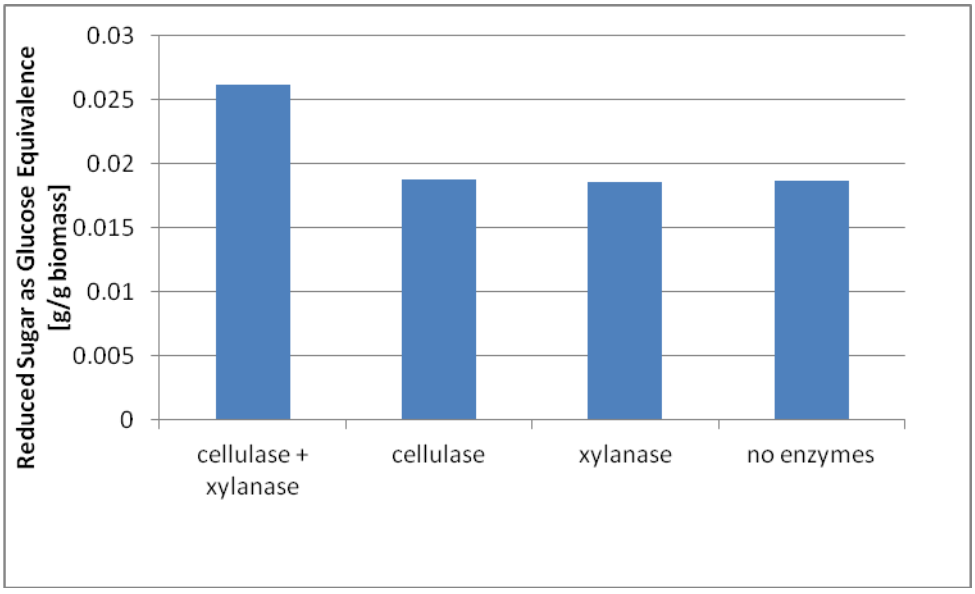


Figure 17. Released sugar of unsterile corn husk using individual crude enzymes

CHAPTER IV

CONCLUSION AND FUTURE STUDIES

With the abundance of lignocellulosic biomass, an effective and economically viable method of depolymerizing lignocellulosic biomass is necessary for bioenergy production. Instead of using purified commercial enzymes, a crude cocktail of lignocellulosic depolymerizing enzymes can be produced using *E. coli*. Three lignocellulosic depolymerizing genes were successfully cloned into pET11a expression vector and transformed into *E. coli* host strains.

High expression of cellulase was observed in *E. coli* BL21 (DE3). Lignin peroxidase and xylanase demonstrated poor expression, regardless of expression by *E. coli* BL21-CodonPlus-(DE3)'s ability to overcome rare codon deficiency. Crude enzymes were produced at approximately 0.18 U/mL, 0.035 U/mL and 0.005U/mL for cellulase, xylanase, and lignin peroxidase activities. Low yields of reduced sugar of corn husk was observed utilizing crude cellulase and xylanase Lignin peroxidase requires separate application from cellulase and xylanase because of addition of H_2O_2 will cause carbohydrate loss, which is counterproductive of this one-pot method.

For future studies, lignin peroxidase and xylanase with lower rare codons can be cloned in pET expression vector to improve enzyme production. Secretion of recombinant gene or autolysis of cells can potentially bypass the tedious extraction process of producing crude enzymes. The one-pot enzymatic hydrolysis can be further to optimized, in terms of enzyme concentration, incubation temperature, and pH.

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APPENDIX A

LITERATURE REVIEW

Properties of lignocellulosic biomass

Cellulose, hemicelluloses, and lignin make up 90% of most plant material[3]. As stated previously cellulose is the most abundant in lignocellulosic biomass, followed by hemicelluloses and lignin (Figure 18). The actual ratio of these components varies by many factors, such as plant species, growth stage of plant and age. Cellulose contributes to the structural and mechanical strengths of plant materials. Cellulose is composed of D-glucose subunits bonded by β-1,4 glycosidic bonds. These long chains are linked by hydrogen bonds and van der Waal bonds forming tight microfibrils.

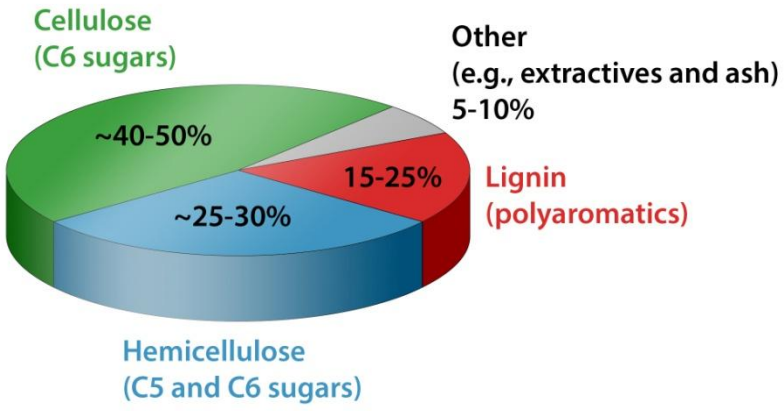


Figure 18. Component of lignocellulosic biomass[22]

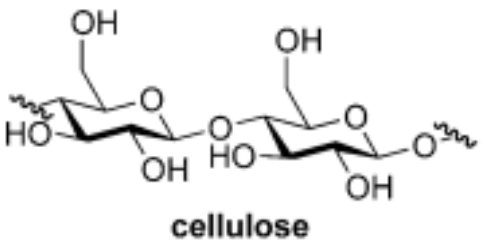


Figure 19. Structure of cellulose[23]

Hemicellulose is composed of various 5- and 6-carbon sugars such as arabinose, galactose, glucose, mannose and xylose, and uronic acid. The sugars are bonded by β-1,4 and β-1,3 glycosidic bonds to form short heterogeneous chains. Xylan is the most common in herbaceous plants while mannan is the most common in woody (hardwood and softwood) biomass.

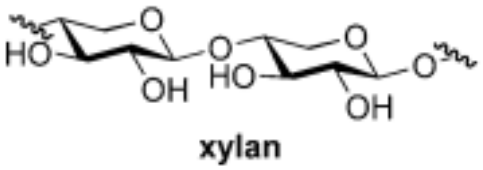


Figure 20. Structure of hemicellulose as xylan[23]

Lignin has a complex heterogeneous marcromolecular structure of phenolic monomers (coniferyl alcohol, coumaryl alcohol, and sinapyl alcohol) linked by ether bonds. Typically more herbaceous plants (grass) have low lignin content and wood biomass have higher lignin content.

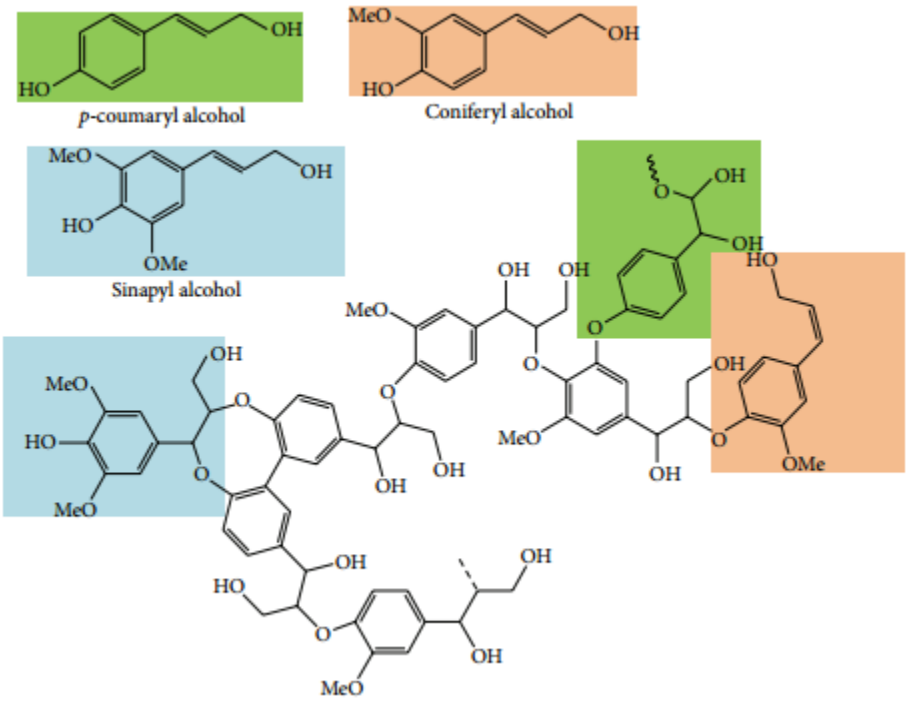


Figure 21. Structure of lignin and phenolic alcohols[24]

Hemicellulose forms tight bonds over the cellulose microfibrils and lignin forms a protective barrier over and in between the carbohydrates, resulting in a material very resistant to microbial degradation. The interactions and ratios among the three components of lignocellulosic biomass is very complex and contribute to their high resistances to degradation by microorganisms.

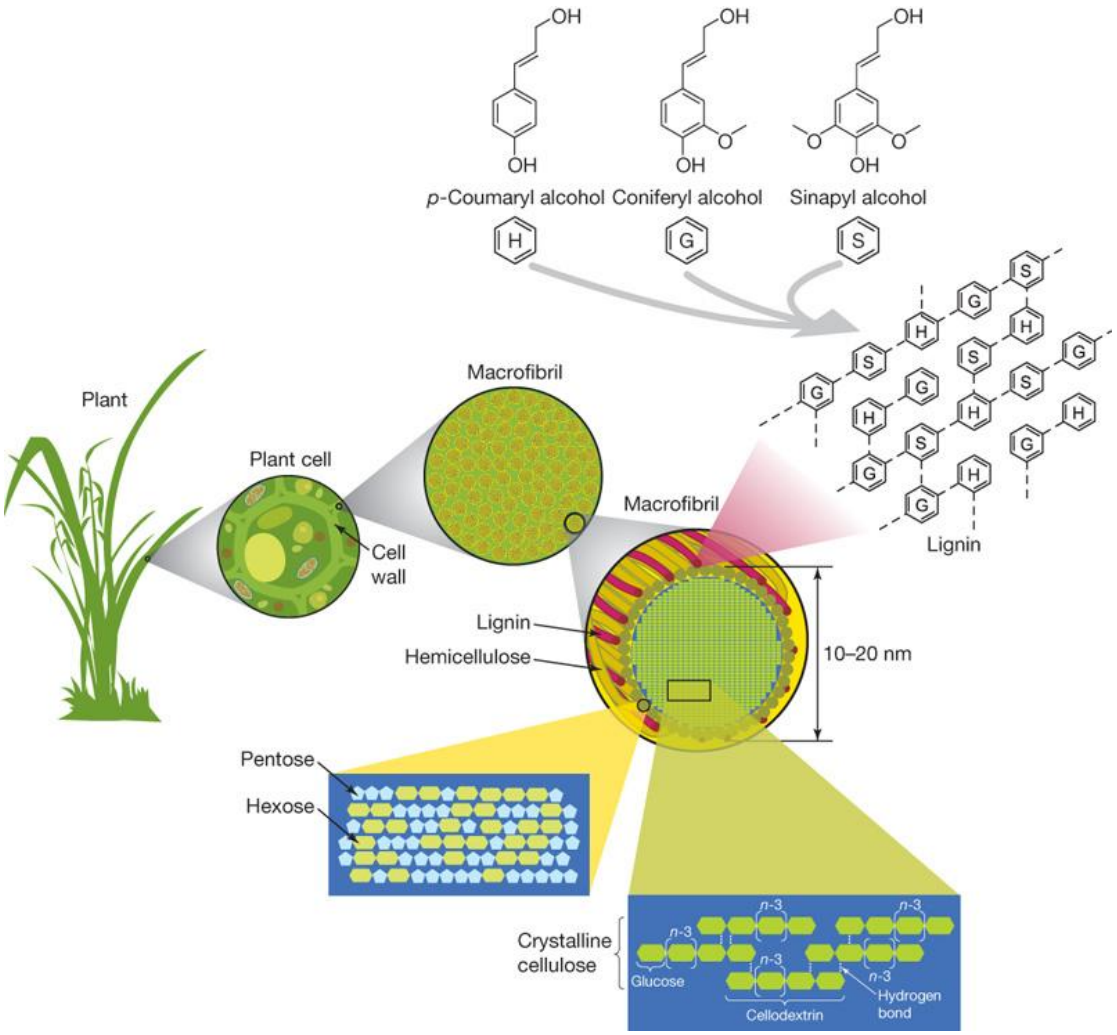


Figure 22. Structure of lignocellulosic biomass[25]

Conversion of lignocellulosic biomass into biofuel

Converting lignocellulosic biomass into biofuel or chemical products typically is a multistep process requiring chemical or physical treatment, enzyme hydrolyzing, and utilization of sugars. Chemical or physical treatment allows higher efficiency of enzymes to hydrolyze carbohydrates into sugars. The sugars are then utilized by microorganisms to form desired products, such as biofuel.

(i) Chemical or physical treatment of lignocellulosic biomass

Chemical or physical treatment of the lignocellulosic biomass swells the lignin structure, reduces crystallinity of cellulose structure, and increases accessibility to cellulose and hemicelluloses[3]. Other factors to consider when selecting a pretreatment method include improving ability to form constitutive sugar, preventing carbohydrate loss, minimizing formation of byproducts that inhibit further hydrolysis and utilization of sugar, being cost efficiency, and minimizing energy input.

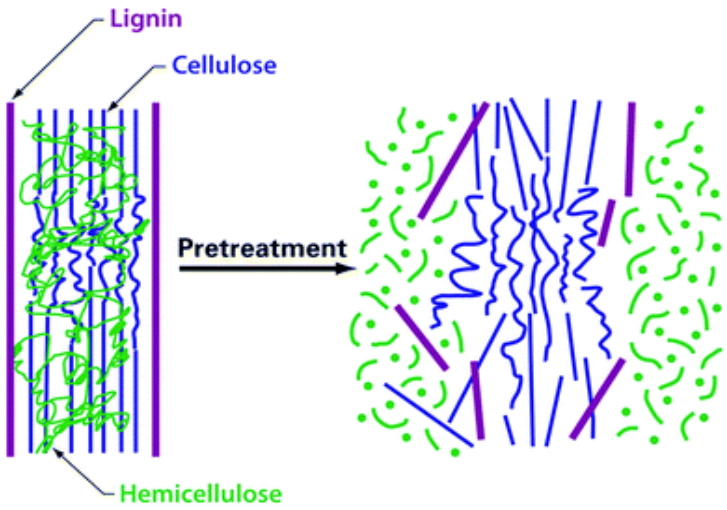


Figure 23. Basic schematics of effect of pretreatment of lignocellulosic biomass[3]

Physical treatment includes milling and ultrasonication and chemical treatment includes acid or alkaline hydrolysis and liquid hot water. No treatments have advantages over all other and comparisons depend on various operating condition and feedstock. There are many combinations of pretreatment methods that improve efficiency but usually at increased cost. Although the treatment of lignocellulosic biomass is necessary

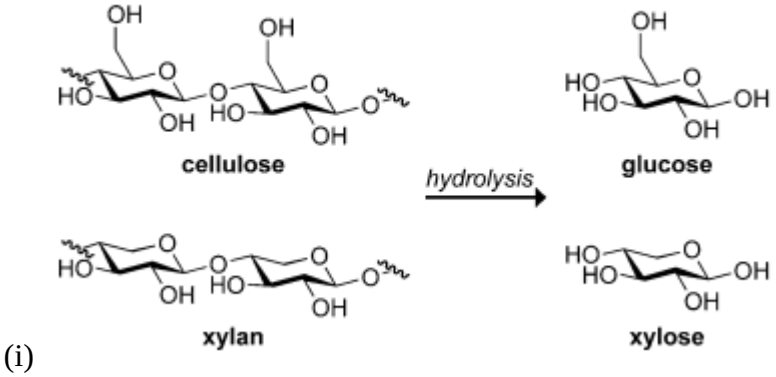
in order to improve efficiency of enzymatic hydrolysis and overall conversion to biofuel, many of these methods have their disadvantages. All physical, thermal and chemical treatment produces toxic byproducts that further inhibit hydrolysis or fermentation of lignocellulosic biomass. There are also biological treatments of lignocellulosic biomass before enzymes hydrolysis. They rely on the natural ability of microorganisms to deconstruct the biomass. Biological treatments are more environmentally and economically viable because they operate at moderate conditions, produce minimal byproducts, and require much less energy input. However biological treatments have much longer retention time and require larger space to cultivate the microorganisms.

(ii) Enzyme hydrolysis of lignocellulosic biomass

After chemical or physical treatment, lignocellulosic biomass is subjected to enzymatic hydrolysis. Lignin depolymerizing enzymes includes lignin peroxidase (LiP), manganese peroxidase (MnP), and laccases. Endoglucanase and b-glucosidase are common cellulases, while xylanase is a more common hemicellulase. Cellulase and hemicellulase are mainly found in fungi, bacteria and protozoa but can also be found in plants and animals. *Trichoderma*, *Apergillus*, *Penicillium* and *Furasruim* species are most commonly used for cellulase production[21]. Lignin degrading enzymes are commonly found in fungi but have been recently identified in bacteria[6,26,27]. Lignin peroxidase found in fungi is more active than those found in bacteria.

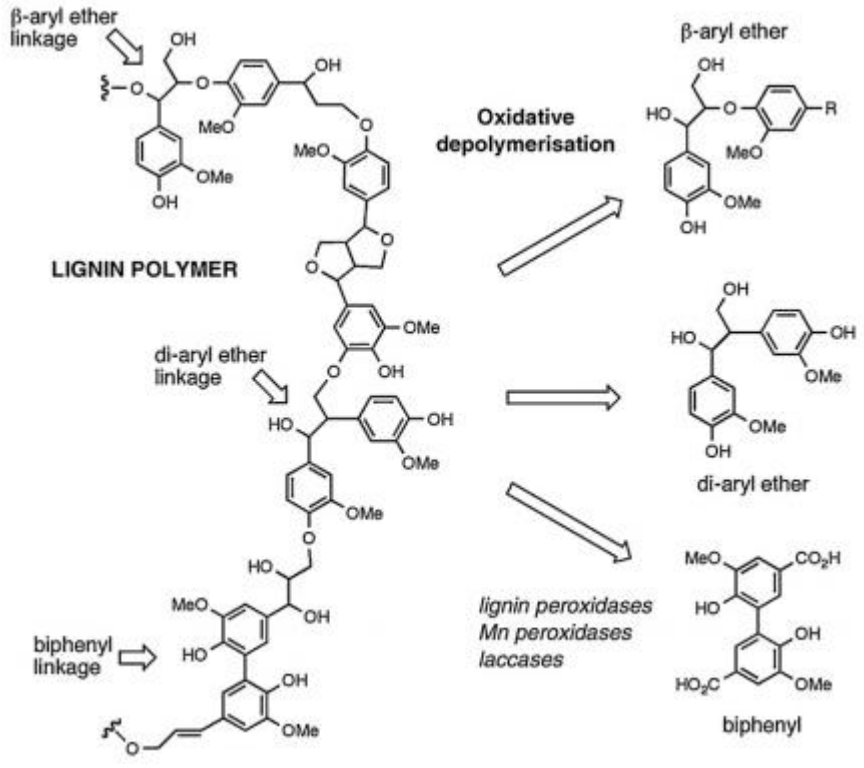
Efficiency of enzymatic hydrolysis is highly sensitive to pH, time, and temperature of substrate. Typical enzyme activities are optimized at 50°C and pH 5.0-6.0

for cellulase and xylanase. Lignin peroxidase is most active at 30°C and pH 3.0. Enzyme activities are listed as the amount that will liberate 1 μmole of glucose, xylose or 3.4-dimethoxybenzyl alcohol per minute or hour at given temperature and pH as specified by the vendor. These purified commercial enzymes are readily available for enzyme hydrolysis at high costs. While hemicelluloses can be easily hydrolyzed under moderate conditions, cellulose needs more extreme conditions to deconstruct. The degree of hydrolysis is highly dependent on cellulose hydrolysis to glucose, since glucose is the main sugar utilized by bioenergy producing microorganisms.



(i)

Figure 24. Enzymatic hydrolysis of cellulose and hemicellulose represented by xylan (i) and lignin (ii)[23,28]



(ii)

Figure 24. Continued[23,28]

A variety of enzymes are required to accommodate extreme conditions of pretreatment methods. Thermal pretreated lignocellulosic biomass requires cooling before hydrolysis with enzymes, because the optimal activities of lignocellulosic depolymerizing enzymes occur at moderate temperatures (30-50°C). Cellulase produced from a novel thermophillic bacterium, *Anoxybacillus* species 527, was found to have optimal activity at 70°C and thermostability up to 100°C[29].

The interaction between the enzymes is also to be considered. Mixture of enzyme can be studied for improved hydrolysis of specific feedstock, based on individual lignin, cellulose and hemicelluloses content. The presence of lignin has been found to decreases

enzyme activity of cellulase (up to 3.5 fold) and xylanase (less than 1.7 fold)[30]. Xylanase provided a synergistic effect on cellulase degradation regardless of low xylan substrate. This indicates that xylanase not only increase access to cellulose by removal of trace xylan but also changes the microfibril structures. A high synergistic effect was also observed with low cellulase and high xylanase loading[31].

(iii) Utilization of hydrolyzed lignocellulosic biomass

Bioenergy products derived from lignocellulosic biomass

Once sugars are released, they can be readily used by different microorganism to produce different precursors of bioenergy compounds, including methanol, ethanol, butanol, and triacylgylceraol (TAG). Fermentation of sugar into ethanol is the most common type of bioenergy production from lignocellulosic biomass. Biogas can also be produced through anaerobic digestion of sugar derived from lignocellulosic biomass[32]. TAG is a major component in fats, oils and lipids derived from plants, animals, fungi, and bacteria. It is also the feedstock for the production of biodiesel, a clean and renewable liquid form of bioenergy. There have been much interest in TAG-accumulating bacteria for their potential for sustainable biodiesel production[33,34,35].

Simultaneous hydrolysis and utilization of lignocellulosic biomass

Hydrolysis and utilization of sugars can occur simultaneously through utilization of selected natural or genetically engineered microorganisms. However, combining these steps may have low efficiency due to byproduct accumulation when depolymerizing

lignocellulosic biomass, such as acetic acid production by *Fusarium oxysporum*[36]. Brown-,white-, and soft-rot fungi are known for their ability to depolymerize lignin, hemicelluloses and cellulose[37]. White rot fungi production of lignin peroxidase and laccase is the most effective at deconstructing of lignin into phenol alcohols. Engineered strains have also been shown to exhibit growth limitations due to alcohol stress[38]. Drawbacks of simultaneous saccharification and utilization includes low hydrolysis rate of lignin and cellulase, growth conditions that need to be carefully monitored, and requirements for large amounts of biomass.

Complete utilization of lignocellulosic biomass by microorganism

Although hemicelluloses contain sugars, they are not easily fermentable. Up to 30% of fermentable sugars in cornstover are xylose[3]. In order to take advantage of the available xylose, many studies have focused on genetically engineering microorganisms to utilize xylose. There are many studies focusing on engineering xylose metabolizing genes into bacteria that otherwise are not able to utilize xylose. Recombinant *Rhodococcus opacus* PD630 has been demonstrated to metabolize xylose and produce microlipids up to 25% cell dry weight, which can be then harvested for biodiesel production[39].

Typically lignin is not deconstructed and not utilized. The goal of deconstructing or removing lignin is mainly to increase accessibility of cellulose and hemicelluloses for hydrolysis. However, recently *R. opacus* PD630 has been identified to have the ability to metabolize depolymerized lignin (phenol alcohol) for growth and microlipid production.

Because these bacteria lack the natural ability to depolymerize lignin, laccase, a known lignin depolymerizing enzyme, was applied to the biomass beforehand[40].

Through adaptive evolution and metabolic engineering, *R. opacus* PD630 have been shown to utilize xylose and phenol alcohol[41]. Co-fermentation of glucose and xylose was successfully achieved by xylanase recombinant *R. opacus* PD630[42]. PD630 is also known to be tolerant of certain lignocellulosic derived inhibitor compounds and can even utilize them as a carbon source for growth and lipid accumulation[12]. The inhibitor compounds are typically produced by pretreatment of lignocellulosic biomass. There has been a study demonstrating increased tolerance of these inhibitor compounds through adaptive evolution[43]. *R. opacus* PD630 is a promising strain for simultaneous hydrolysis and utilization of lignocellulosic biomass because of its ability to degrade many recalcitrant compounds.